ADDITION OF SULFAMETHOXAZOLE TO SELECTIVE MEDIA AIDS IN THE RECOVERY OF *CAMPYLOBACTER* SPP. FROM BROILER RINSES

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ABSTRACT

The association of Campylobacter *spp. with raw poultry products has been well established. Campy-Line agar (CLA) is a recently developed selective culture medium that allows very few non-*Campylobacter *colonies to grow. The few contaminants able to grow on CLA from typical broiler chicken carcass rinses were identified and found to be sensitive to the antimicrobial agent sulfamethoxazole (SMX). The purpose of our experiment was to determine the influence of SMX on recovery of* C. jejuni *from broiler carcass rinse samples when added to Campy-Cefex agar or CLA. Cefex and CLA were prepared with and without the addition of 25 mg SMX/L media. Broiler carcass rinse samples (post-pick,* n = *80 and post-chill,* n = *80) were obtained from eight different commercial processors across the United States and were surface plated on the selective agars. Both Cefex and CLA with and without SMX recovered similar populations of* Campylobacter; *however, significantly fewer contaminants were observed on the Cefex with added SMX and the CLA with or without SMX compared to normal Cefex. The more selective CLA with SMX had no contaminants present from this sample type, which simplified enumeration. Addition of SMX should be considered for increasing selectivity of* Campylobacter-*specific media.*

PRACTICAL APPLICATIONS

The addition of sulfamethoxazole to *Campylobacter*-selective agar can reduce the number of contaminants on the agar and facilitate enumeration of *Campylobacter* colonies.

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INTRODUCTION

Campylobacter is one of the leading causes of human bacterial gastroenteritis in the United States and around the world (Tauxe 1992; Taylor 1992; Altekruse *et al.* 1999; Schlundt *et al.* 2004; Stern *et al.* 2005). The association of *Campylobacter* spp. with raw poultry products has been well established (Skirrow 1991; Shane 2000; Corry and Atabay 2001). Efforts have been ongoing to reduce the amount of *Campylobacter* present in raw poultry and these interventions have been targeted at both the production farm and processing plant environments. In order to evaluate the effect of interventions on *Campylobacter* populations associated with poultry, it is necessary to utilize bacteriological methods that allow numerical estimations of pathogen populations present in the sample (quantitative data) to be obtained. Because many interventions may only reduce populations of *Campylobacter* and not completely eliminate it, quantitative methods are necessary to determine the effectiveness of treatment. Classic microbiological enrichment procedures may sometimes be more sensitive, but generally, they only allow qualitative data to be gathered. The most probable number (MPN) technique combines growth in enrichment broth with multiple tubes at several dilutions to obtain numerical estimates, essentially combining the sensitivity of enrichment with the ability to quantitate. The method is very labor intensive however, and in a collaborative study conducted by the Agricultural Research Service and Food Safety Inspection Service laboratories (Line *et al.* 2001), the difference between recovery of *Campylobacter* by the MPN method and direct plating on Campy-Cefex agar (Stern *et al.* 1992) was not significant.

Direct plating on Campy-Cefex has been widely used in a variety of studies to determine the levels of *Campylobacter* contamination associated with poultry samples (Shih 2000; Musgrove *et al.* 2003; Stern and Robach 2003); however, sometimes there are issues with non-*Campylobacter* contaminants that can also grow on the plates and make counting of *Campylobacter* colonies difficult. The Campy-Cefex plates contain blood, and hence, are dark red in color. *Campylobacter* appears as light gray or clear colonies (similar in appearance to water droplets) on the surface of the agar. There is therefore little contrast between the colony color and the background agar, making enumeration difficult. There is a need for a more selective and differential agar for enumeration of campylobacters associated with broiler chicken carcasses and other sample types.

A new selective agar called Campy-Line agar (CLA) (Line 2001), was developed, which incorporated triphenyltetrazolium chloride (TTC) into the agar. Campy-Line agar contains no blood and is translucent. Campylobacters are able to reduce the colorless TTC in the CLA to red-colored formazan compounds, resulting in a dark red- or magenta-colored colony that is easily distinguishable. Reduction of TTC to formazan compounds is not unique to C*ampylobacter*, however. Some other microorganisms may also produce a red-colored colony on the agar. Although selective antimicrobial agents in the CLA serve to eliminate most non-*Campylobacter* microbes, we observed five primary contaminants from commercial poultry carcass rinses that would grow on the CLA and could confound results. These contaminants were identified as *Sphingomonas paucimobilis, Acinetobacter baumannii, Brevundimonas diminuta, Ochrobactrum* spp. and *Flavobacterium odoratum*. Screening by a traditional minimal inhibitory concentration technique using 23 different antimicrobial agents revealed all of the contaminants to be sensitive to sulfamethoxazole (SMX).

Sulfamethoxazole is an antibacterial sulfonamide. It acts to prevent the formation of dihydrofolic acid, a compound that bacteria must be able to make in order to survive. SMX is less effective as a single antimicrobial agent today than it was when first approved by the Food and Drug Administration in 1961 due to development of antimicrobial resistance in many microorganisms (Anon. 2004b). SMX is now used primarily in conjunction with trimethoprim, creating a synergistic interaction between the antimicrobial agents that is more inhibitory to bacterial growth (Anon. 2004a). Trimethoprim is a component of CLA. We conducted preliminary experiments using SMX and determined that 25 mg/L in CLA was sufficient to prevent growth of any of the primary contaminants we had identified, yet *Campylobacter* growth was unaffected at these concentrations. The purpose of this study was to determine the influence of SMX on recovery of *Campylobacter* spp. from commercial broiler carcass rinse samples when added to CLA or Campy-Cefex agar.

MATERIALS AND METHODS

Four different selective media were prepared: Campy-Cefex, CLA, and both of these agars with an added 25 mg SMX/L. Commercial broiler carcasses (post-pick, $n = 80$ and post-chill, $n = 80$) were obtained from eight different poultry processors from across the United States during the course of about 1 year. Carcasses were rinsed in the plant in 400 mL buffered peptone and the rinse samples were maintained at less than 8C during overnight shipment to our lab. Samples were analyzed promptly upon receipt. For each of the four selective media, a total volume of 1.0 mL of sample was surface plated on four plates (0.25 mL each plate). This was done to increase the sensitivity of the direct plating procedure. The counts from these four plates were combined to get a count per mL estimate. An additional two duplicate plates were each inoculated with 0.1 mL and the colony counts from these two plates were averaged. All plates were incubated for 48 h at 42C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Following incubation, plates were inspected for growth. Typical *Campylobacter* colonies were enumerated and confirmed using microscopic wet mount and latex agglutination for confirmation as necessary. Typical colonies (3–4) were picked from a plate and pooled in 25 μL of 0.85% saline. This slurry was used to prepare microscopic wet mounts and to confirm the presence of *Campylobacter* spp. using the latex agglutination assay (Integrated Diagnostics, Inc., Baltimore, MD). Non-*Campylobacter* colonies were also enumerated on the plates. Mean Log_{10} values were calculated and significant differences between groups were determined using the *t*-test or Mann–Whitney rank sum test as appropriate (SigmaStat statistical software, Jandel Scientific Software, San Rafael, CA). Plates with no colonies were entered as zeros in calculations and assigned a Log_{10} value of zero for purposes of statistical comparison.

RESULTS

Populations of *Campylobacter* spp. as well as contaminants were much greater on the post-pick samples (Table 1) than on the further processed post-chill samples (Table 2). All post-pick samples ($n = 80$) were positive for *Campylobacter* spp. on all the media tested. *Campylobacter* was recovered from only 40 of the 80 post-chill samples when results from all the plating media were combined (Table 3). This trend was also reflected in the number of poultry processing plants producing *Campylobacter* positive samples. No *Campylobacter* spp. was recovered by direct plating from the post-chill samples from three of the eight plants analyzed.

There was much variation in the levels of *Campylobacter* in the rinse samples from the eight different plants. Post-pick *Campylobacter* spp. populations ranged from zero to Log_{10} 6.0, while post-chill *Campylobacter* spp. populations varied between zero and Log_{10} 3.5. Within groups of 10 carcass rinses from individual plants, there were always *Campylobacter* negative post-chill samples interspersed with positive post-chill samples whenever *Campylobacter* positive samples were detected. There was no correlation between the *Campylobacter* levels found in the post-pick samples and the populations determined in the post-chill samples from the same plant ($P > 0.05$). Interestingly, the plant with the most *Campylobacter* in the post-chill samples (Plant 6) did not have the greatest *Campylobacter* populations in the post-pick samples, demonstrating probable differences in processing conditions between the eight plants.

There was no significant difference ($P < 0.05$) in *Campylobacter* spp. recovery between the four plating media for either post-pick or post-chill samples. However, significantly fewer contaminants ($P > 0.05$) were observed

TABLE 1.
RECOVERY OF *CAMPYLOBACTER* SPP. AND NON-*CAMPYLOBACTER* CONTAMINANTS FROM POST-PICK BROILER CARCASS RINSE SAMPLES ($n = 80$) ON CLA AND CAMPY-CEFEX AGARS WITH AND WITHOUT ADDED SULFAMETHOXAZOLE (25 mg/L)

Poultry plant	Mean $\log_{10}$ cfu *Campylobacter* spp./mL				Mean $\log_{10}$ cfu non-*Campylobacter* contaminants/mL			
	Cefex	Cefex + SMX	CLA	CLA + SMX	Cefex	Cefex + SMX	CLA	CLA + SMX
1	2.88	2.69	2.74	2.64	0.39	0.48	0.1	0
2	4.01	3.79	3.83	3.75	1.64	0.56	0	0
3	4.29	4.20	4.19	4.15	4.13	2.45	0	0
4	3.52	3.59	3.53	3.56	1.88	0.60	0	0
5	3.51	3.17	2.98	3.11	2.88	1.60	0	0
6	2.80	3.47	3.32	3.15	3.25	2.04	0	0
7	2.86	3.46	3.54	3.50	2.89	3.01	3.21	0
8	2.60	2.82	3.06	2.95	NA	2.75	2.21	0
Mean (SD)	3.31 (0.99)	3.40 (0.82)	3.40 (0.77)	3.35 (0.82)	2.44^{a} (1.39)	1.68^{b} (1.24)	0.69^{c} (1.21)	0^{d} (0)

^{abcd}Means in the same row with different superscripts are significantly different ($P < 0.05$).
CLA, Campy-Line agar; SMX, sulfamethoxazole; NA, data not available.

TABLE 2.
RECOVERY OF *CAMPYLOBACTER* SPP. AND NON-*CAMPYLOBACTER* CONTAMINANTS FROM POST-CHILL BROILER CARCASS RINSE SAMPLES (n = 80) ON CLA AND CAMPY-CEFEX AGARS WITH AND WITHOUT ADDED SULFAMETHOXAZOLE (25 mg/L)

Poultry plant	Mean $\log_{10}$ cfu *Campylobacter* spp./mL				Mean $\log_{10}$ cfu non-*Campylobacter* contaminants/mL			
	Cefex	Cefex + SMX	CLA	CLA + SMX	Cefex	Cefex + SMX	CLA	CLA + SMX
1	0.74	0.1	0.2	0.31	2.53	0	0	0
2	0	0	0	0	0.07	0.24	0.21	0
3	0.38	0.41	0.27	0.19	0	0.94	0	0
4	0	0	0	0	0	0	0	0
5	0.19	0.15	0.27	0.26	1.51	0.2	0	0
6	1.30	1.58	1.27	1.05	0.54	0.1	0	0
7	0	0.23	0.34	0.26	1.95	1.32	1.05	0
8	0	0	0	0	NA	0.28	0.04	0
Mean (SD)	0.33 (0.58)	0.31 (0.64)	0.29 (0.64)	0.26 (0.57)	0.94^{a} (1.16)	0.36^{b} (0.74)	0.16^{b} (0.50)	0^{b} (0)

[ab]Means in the same row with different superscripts are significantly different ($P < 0.05$).
CLA, Campy-Line agar; SMX, sulfamethoxazole; NA, data not available.

TABLE 3.
NUMBER OF DIRECTLY PLATED POST-PICK AND POST-CHILL *CAMPYLOBACTER* POSITIVE SAMPLES BY PLANT AND BY MEDIA TYPE

Plant	Post-pick samples					Post-chill samples				
	Cefex	Cefex + SMX	CLA	CLA + SMX	All 4 Media	Cefex	Cefex + SMX	CLA	CLA + SMX	All 4 Media
1	10	10	10	10	10	8	1	2	4	9
2	10	10	10	10	10	0	0	0	0	0
3	10	10	10	10	10	5	5	2	2	6
4	10	10	10	10	10	0	0	0	0	0
5	10	10	10	10	10	3	5	2	4	10
6	10	10	10	10	10	9	9	8	7	10
7	10	10	10	10	10	0	3	4	3	5
8	9	10	10	10	10	0	0	0	0	0
Total	79	80	80	80	80	25	23	18	20	40

from post-chill samples on the Campy-Cefex with added SMX or CLA (with or without SMX) as compared to normal Campy-Cefex. There were significant differences ($P > 0.05$) in number of contaminants growing on the selective media between all the plating media types for the post-pick samples. Non-*Campylobacter* spp. contaminating strains differed between plants as evidenced by the breakthrough of contaminants resistant to the antimicrobials in CLA that were observed from plants 7 and 8 (Table 1). Fortunately, the modified CLA containing SMX was able to eradicate these contaminants resulting in the detection and enumeration only of *Campylobacter* spp. colonies on the CLA + SMX plates. No contaminants were isolated on the CLA with SMX from either sample type ($n = 160$) due to the increased selectivity of the CLA as compared to Cefex and the added effect of the SMX antimicrobial. The absence of non-*Campylobacter* spp. contaminants on the CLA + SMX plates greatly facilitated counting of the colonies as did the contrasting color of the colonies on the agar surface.

DISCUSSION

As expected, *Campylobacter* populations were found to be lower in the post-chill samples than in the post-pick samples because of the additional exposure of the pathogens to microbiologically stressful or bacteriocidal conditions. The post-chill samples were likely exposed to inhibitory chlorine levels in the chill tank as well as to tri-sodium phosphate or other inhibitory compounds in the pre-chill washes. In addition, exposure to oxygen and drying of the carcass post-chill combine to increase stress on the organism and reduce viable *Campylobacter* populations (Nachamkin 2001; Stern *et al.* 2001).

Other studies have demonstrated no significant difference ($P > 0.05$) between CLA and Campy-Cefex media in recovery of campylobacters from broiler carcass rinse samples and a high correlation between the two media (Line 2001; Siragusa *et al.* 2004). Line and Berrang (2005) also found a high correlation between the two agars when recovering *Campylobacter* from a variety of poultry samples including feathers, skin, crop, ceca and colon. Pearce *et al.* (2003) found a significantly higher recovery rate for *C. jejuni* and *C. coli* from swine carcasses and processing facility environmental samples by direct plating on CLA as compared to Campy-Cefex agar or Bolton broth enrichment. Oyarzabal *et al.* (2005), however, found CLA to give significantly lower *Campylobacter* counts from carcass rinses compared to direct plating on Campy-Cefex, mCampy-Cefex, mCCDA, Karmali or Campy media. Interestingly, Oyarzabal *et al.* (2005) found that the most prevalent contaminant in their study was *Acinetobacter baumannii*, which grew on all plates except for CLA. Line (2001) demonstrated that this same contaminant (*A. baumannii*) is a frequent contaminant on CLA, and this was one of the reasons SMX was investigated for addition to CLA in the current study. Inability of this contaminant to grow on standard CLA in the Oyarzabal media comparison study indicates that the CLA possibly was formulated incorrectly and could have negatively impacted recovery of *Campylobacter* on the CLA in that study.

There is an inherent risk in any microbiological analysis especially when attempting to isolate stressed microorganisms. If the plating medium is too selective and the antimicrobial agents are too harsh, they may eliminate unwanted contamination, but they may also impair recovery of sub-lethally injured target organisms. Conversely, if the medium is not selective enough, unwanted contaminants may grow profusely on the plate, making it difficult, if not impossible, to detect the target microorganism. A balance must be achieved where the selective medium does not significantly affect recovery of the target microorganism, yet sufficiently inhibits growth of unwanted competitors on the plate. The addition of SMX to Campy-Cefex and CLA helped to reduce contamination on the plates without significantly affecting *Campylobacter* spp. recovery. In the case of CLA + SMX, the competitors were eliminated completely, making the medium easier for technicians to utilize. The addition of SMX should be considered for increasing selectivity of *Campylobacter*-selective media for this sample type.

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